

Video Article

# Milk Collection in the Rat Using Capillary Tubes and Estimation of Milk Fat Content by Creamatocrit

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## Abstract

Milk, as the sole source of nutrition for the newborn mammal, provides the necessary nutrients and energy for offspring growth and development. It also contains a vast number of bioactive compounds that greatly affect the development of the neonate. The analysis of milk components will help elucidate key factors that link maternal metabolism and health with offspring growth and development. The laboratory rat represents a popular model organism for maternal studies, and rat milk can be used to examine the effect of various maternal physiological, nutritional, and pharmacological interventions on milk components, which may then impact offspring health. Here a simple method of manually collecting milk from the lactating rat that can be performed by a single investigator, does not require specialized vacuum or suction equipment, and provides sufficient milk for subsequent downstream analysis is described. A method for estimating the fat content of milk by measuring the percentage of cream within the milk sample, known as the creatomatocrit, is also presented. These methods can ultimately be used to increase insight into maternal-child health and to elucidate maternal factors that are involved in proper growth and development of offspring.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/53476/>

## Introduction

Milk is the sole source of nutrition for newborn mammals, providing energy and nutrients for infant growth and development<sup>1,2</sup>. While milk mainly consists of cells, lipids, and protein<sup>1</sup>, it also contains a plethora of bioactive compounds that modulate early life development of offspring including enzymes, carbohydrates, hormones, antibodies, growth factors, cytokines, exosomes, microvesicles, and small RNAs such as microRNA<sup>1,2</sup>. The fundamental role of maternal milk in the establishment of offspring immune and intestinal health<sup>3</sup>, coupled with evidence that breastfed infants are less susceptible to disease<sup>2</sup>, highlights the importance of identifying the milk constituents associated with disease processes in early life and the molecular mechanisms involved in their actions. The developing rat is a popular model for investigating the effect of various nutritional, physiological, and chemical interventions on early-life development<sup>4</sup>. The analysis of rat milk may therefore provide novel insight into maternal and offspring health.

Current scientific advances now provide increasing opportunities for in-depth investigations of the effects of specific milk constituents on health and disease. For example, sequencing of milk bacterial profiles has elucidated their role in early intestinal colonization of the infant gut<sup>5</sup>, mass spectrometry analysis of milk oligosaccharides have provided insight into the alteration of milk oligosaccharide profiles via maternal diet<sup>6</sup>, and deep sequencing of microRNA secreted in the fat globules of breast milk highlights possible roles in gene transcription, metabolism, and immune function<sup>7</sup>.

Rat models represent one of the most popular model organisms used in maternal studies<sup>8,9</sup>. One advantage is their short gestation and lactation periods, lasting only approximately 21 days each; therefore the total time from the start of pregnancy to lactation represents a short period of time in which valuable data can be generated. The larger size of rats compared to mice, in the context of milk collection, may provide a significant advantage with respect to volume of milk and ease of milk collection; milk production in the mouse, for example, seems to be dependent on total body weight with heavier mice producing more milk<sup>10</sup>.

Here, a general description for the manual collection of milk from lactating rats is provided. This protocol requires minimal equipment, is non-invasive, inexpensive, and can be used to collect adequate volumes of milk for further downstream analyses. In brief, the dam is anesthetized with isoflurane, milk letdown is stimulated by oxytocin, and milk is collected into capillary tubes via manual expression of the milk. Finally, as two major components of milk are fat and proteins, a brief description of estimating milk fat content using creatomatocrit measurements<sup>11</sup> and quantification of total protein concentration using a standard protein assay is presented.

## Protocol

This protocol was approved by the University of Calgary Animal Care Committee and conformed to the *Guide for the Care and Use of Laboratory Animals*.

### 1. Separate Dam from Offspring

1. Separate the dam from her offspring for a minimum of 5 min prior to milking<sup>12</sup>.  
NOTE: The dam can be milked up to 5-6 hr after separation<sup>1,6,13</sup>, however periods of separation longer than 4 hr may alter milk composition<sup>14</sup>. While separation time does not appear to impact milk collection volume<sup>12</sup>, it is advised that a consistent separation time be maintained throughout the study. Milk composition may change throughout lactation<sup>15</sup>, therefore attempts should be made to keep the day of milk collection consistent. Maximum milk production is suggested to occur on lactation day 14<sup>12</sup>.
2. Using a warming chamber, ensure the pups are able to maintain proper body temperature without the presence of their mother for the duration of the milking procedure.  
NOTE: In the study presented below, milking was performed at weaning, when the dams were approximately 22 weeks old and the offspring 21 days old, therefore no warming chamber was used.

### 2. Set-up and Preparation

1. Collect all materials required for the milking procedure.  
NOTE: All materials can be found in the materials and equipment table.
2. Place a heating pad on the bench where milking will take place and cover the pad with an absorbent bench under-pad.
3. Set up the anesthetic system. Ensure that the system has sufficient oxygen and isoflurane prior to beginning. Attach the anesthetic mask that will be used for initial anesthesia induction to the machine. Place the mask that will be used for anesthesia maintenance nearby if different than the initial anesthetic mask.
4. Attach a 25 G needle to a 1 ml syringe for oxytocin injection using aseptic technique.
5. Turn on the heating pad so that maternal body temperature is maintained during the milking procedure. NOTE: Monitor the temperature of the heating pad to ensure the pad does not become too hot and cause burns. Alternatively, use a heat source, such as a heated surgical table, that can be set to a specific temperature.

### 3. Anesthetize the Dam Using Isoflurane

1. Open the oxygen tank and turn the flow to 1 L (1,000 cc) per min. Turn on the flow of isoflurane and set to 5%. CAUTION: Avoid direct inhalation of anesthetic and prevent accumulation of anesthetic vapors.
2. Anesthetize the dam.
3. Switch over to the maintenance mask if required, placing the dam supine on the absorbent bench pad. Confirm anesthetization by lack of pedal reflex.
4. Reduce the flow of isoflurane to 2-3% for maintenance of anesthesia. Continually monitor dam throughout the procedure to ensure depression of respiration does not occur. NOTE: Once under anesthesia, the dam's eyes should be protected using a sterile eye lubricant to prevent the eyes from drying out or becoming scratched.

### 4. Oxytocin Injection

1. Ensure the oxytocin (20 USP Units/ml) has not passed its expiry date. Disinfect the vial of oxytocin with a sterile alcohol wipe/alcohol cleansing pad.
2. Using aseptic technique, draw up 2 IU (0.1 ml) of oxytocin into the syringe. Use a new needle and syringe for each dam that will be milked. NOTE: Oxytocin doses generally range from single injections of 1 to 5 IU<sup>1,6,12,16</sup>. Alternatively, a dose of 4 IU/kg body weight can be used<sup>12</sup>. A single dose of 2 IU can be repeated once if difficulty milking is encountered.
3. Inject the oxytocin intraperitoneally. Insert the needle into the lower right quadrant of the abdomen with the needle pointing towards the head, at an angle of 15-30°, about 0.5 cm deep.
4. Pull back on the plunger to ensure negative pressure prior to injection. If any fluid (blood, urine, intestinal contents, etc.) is aspirated into the syringe, remove the needle and attempt the injection with a new needle and syringe. If no fluid is aspirated inject the oxytocin and discard the needle and syringe immediately into a biohazard container.
5. Wait approximately 5-15 min for the oxytocin to stimulate milk letdown.

### 5. Preparation of Milking Sites

1. Choose the sites/teats from which milk will be collected. Milk can be collected from any teat<sup>12</sup>.
2. Gently remove the fur around the teats to be milked with the trimmers, as fur may cause difficulty in sample collection due to wicking of the milk. Be gentle - the skin around the teats is extremely sensitive and may be dry and as such is susceptible to scratches and tears.
3. Sterilization of the teat is not necessary, but optionally, clean the teat with lukewarm water after the fur is removed. Prepare at least two sites as more than one site may be required for milk collection.  
NOTE: If the milk analysis includes microbial profiling, the teat area may require sterilization with iodine<sup>5</sup>.

## 6. Milk Collection

1. Gently squeeze the base of the teat, manually expelling the milk for collection.  
NOTE: If the milk analysis includes microbial profiling, the first few drops of milk should be discarded.
2. Collect the milk droplets into a capillary tube, filling the capillary tube. Capillary tubes that accommodate larger volumes (e.g., 50  $\mu$ l) ease the process.  
NOTE: If difficulty in collecting milk is encountered, a second dose of oxytocin can be administered. It is recommended the dose not exceed 4 IU total.
3. Dispense the milk from the capillary tube into a sterile microcentrifuge tube by touching the end of the tube that was used to draw milk from the teat to the side of the microcentrifuge tube - observe the milk being drawn out of the capillary tube via capillary action.  
NOTE: 'Blow out' the milk that is not drawn into the tube using an 18 G needle attached to a 1 ml syringe.
4. Continually monitor the dam for signs of pain or respiratory depression and adjust the flow of isoflurane accordingly.
5. Continue to collect milk as described in this section until sufficient milk has been collected for the chosen milk analysis. For the creatmatocrit and protein concentration determination described below, collect 0.25 ml of milk.  
NOTE: Use a different milking site if milk letdown slows or the chosen site does not expel milk sufficiently. A maximum of approximately 2.5 ml of milk per animal can be collected<sup>17</sup>, or up to 0.5 ml per teat. The authors recommend that total time under anesthetic be limited to approximately 45-60 min, or 45 min of milking.
6. Collect milk into a microhematocrit tube for creatmatocrit measurement from fresh milk samples, and seal the end of the tube with clay sealant. Label the microhematocrit tube with the dam ID and store the milk sample upright.
7. When milking is complete, turn off the flow of isoflurane and oxygen. Remove the mask from the dam and continue to monitor dam until awake. It is recommended that if not fully conscious, the dam should be placed on an absorbent bench pad during the recovery period, rather than directly on the cage bedding, to prevent the bedding from being aspirated or scratching the dam's eyes during recovery.  
NOTE: Do not leave the dam unattended until it has regained sufficient consciousness to maintain sternal recumbancy.
8. If no further analyses are required, freeze milk at -80 °C. Others have suggested that milk can be stored for up to 3 hr at 4 °C or 5 months at -20 °C<sup>18</sup>.

## 7. Creamatocrit Measurement

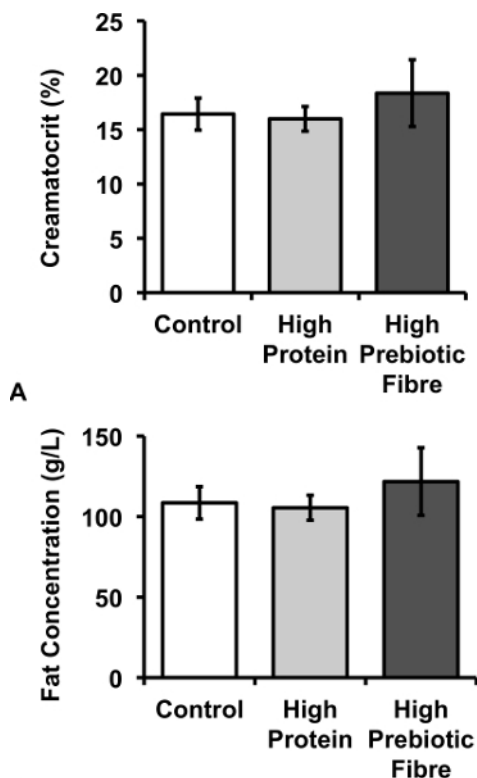
1. Estimate the fat content in the milk by calculating the milk creatmatocrit (the percentage of cream in the milk sample)<sup>19</sup>.  
NOTE: Measurements with human milk have demonstrated that either fresh or frozen milk can be used for a creatmatocrit measurement, however fresh milk is more highly correlated ( $r = 0.92$  versus  $r = 0.90$ ) with lipid concentration<sup>11</sup>. The use of fresh or frozen milk for creatmatocrit measurements should be kept consistent across the study, as thawed milk is associated with a small decrease in creatmatocrit values<sup>11</sup>.
2. For fresh milk, collect a sample of milk from the teat into a microhematocrit tube; fill at least  $\frac{3}{4}$  full (approximately 15-20  $\mu$ l). Alternatively, draw fresh milk from the collected sample into the capillary tube after mixing well. Seal the end with clay sealant.
3. Place the capillary tube into the hematocrit spinner, with the sealed end pointing towards the outside, ensuring the centrifuge is balanced.
4. Begin the hematocrit spin (120 sec at 13,700 x g).  
NOTE: Spin time or speed may change depending on the model of centrifuge used.
5. Remove the tube from the centrifuge after the spin is complete and perform the measurements for calculating the creatmatocrit. Observe the sample's separation into a cream layer and a clear layer.
6. Measure and record the total length of fluid in the tube and the length of the fat (cream) layer using calipers or a ruler.  
NOTE: The creatmatocrit is expressed as the percentage of the cream layer within the the milk sample<sup>19</sup>, calculated as (length of cream layer/ total length of milk column) x 100 (**Figure 1A**). Calculate fat concentration and energy values from the creatmatocrit measurement as follows: Fat concentration (g/L) = (creamatocrit (%)-0.59)/0.146 (**Figure 1B**)<sup>19</sup>; Energy value (kcal/L) = 290 + (66.8\*creamatocrit (%)) (**Figure 1C**)<sup>19</sup>.

## 8. Protein Concentration Determination

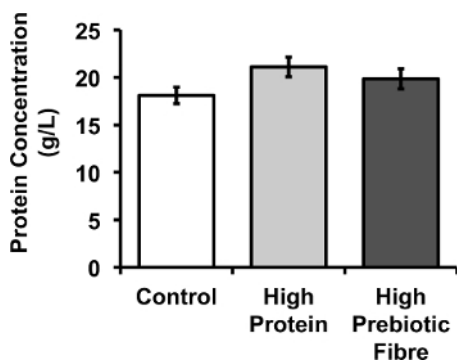
1. Using Bovine Serum Albumin as a protein standard, determine total milk protein concentration using a standard protein assay, such as a Lowry protein assay<sup>6</sup>.  
NOTE: Dilution of the milk may be necessary for the milk protein measurements to fall within the standard curve of the assay.

## Representative Results

Milk was collected as described at weaning from Wistar dams (approximately 22 weeks old, weighing 350 to 400 g) that consumed a control (AIN-93G,  $n = 5$ ), high protein (40% casein wt/wt,  $n = 5$ ), or high prebiotic fibre (21.6% wt/wt, 1:1 ratio of oligofructose and inulin,  $n = 4$ ) diet throughout pregnancy and lactation. The oxytocin dose was 2 IU. Milk was collected using capillary tubes, and one tube was spun using a hematocrit spinner to determine creatmatocrit (**Figure 1A**), which was then used to estimate fat concentration and energy value according to: Fat concentration (g/L) = (creamatocrit (%)-0.59)/0.146 (**Figure 1B**); Energy value (kcal/L) = 290 + (66.8\*creamatocrit (%)) (**Figure 1C**)<sup>19</sup>. Milk protein concentration was determined using the Bio-Rad DC protein assay (**Figure 2**). There were no differences in creatmatocrit ( $p = 0.674$ ), fat concentration ( $p = 0.674$ ), energy value ( $p = 0.674$ ), or protein concentration ( $p = 0.127$ ) based on maternal diet (one-way ANOVA).



**Figure 1. Milk creatatocrit, fat concentration, and energy value.** Milk samples were collected at weaning from Wistar dams on Control (n = 5), High Protein (n = 5), or High Prebiotic Fibre (n = 4) diets throughout pregnancy and lactation. Creatatocrit measurements (A) were used to calculate milk fat concentration (B) and energy value (C). [Please click here to view a larger version of this figure.](#)



**Figure 2. Total milk protein concentration.** Milk samples were collected at weaning from Wistar dams on Control (n = 5), High Protein (n = 5), or High Prebiotic Fibre (n = 4) diets throughout pregnancy and lactation. Total protein concentration was determined using the Bio-Rad DC protein assay. [Please click here to view a larger version of this figure.](#)

## Discussion

Investigations into maternal milk components have increased as interest in early life development research rises. As the sole source of nutrition during the neonatal period, the bioactive compounds in milk are essential for ideal growth and development, especially in the context of intestinal and immune health<sup>3</sup>. The method presented here is a simple, non-invasive method of collecting milk from the lactating rat in amounts sufficient for downstream analysis, such as oligosaccharide profiling<sup>6</sup>. The method requires no specialized vacuum equipment and can be performed by a single person.

While this protocol is intended for use at a single time point during lactation, others have performed serial milk collections throughout their studies<sup>12,15</sup>. However, as serial milking may affect milk composition<sup>17</sup>, it is advised that researchers determine the frequency and number of milk collections that best suit their study, based on the outcome of interest. Additionally, while others have used injectable anesthetics during milking procedures in the rat<sup>14,17</sup>, this protocol involves anesthetization of the rat using isoflurane. Isoflurane is an inhalable anesthetic, and its advantages include rapid induction and recovery rates<sup>20</sup> and the ability to easily adjust the time the animal is anesthetized. However, in mice, isoflurane may result in decreased milk yield in comparison to injectable anesthetics<sup>10</sup>, although this does not seem to have been tested in rats.

Furthermore, protocols involving vacuum suction may not require anesthetic, and may result in higher milk yield depending on the efficiency of the suction apparatus<sup>12</sup>. However, if proper suction does not occur, milk yield will decrease.

It is possible for the researcher to encounter difficulty in manual expression of milk, which can prolong time under anesthesia or decrease the milk yield. If difficulty in milking is encountered, some possibilities are: 1) sufficient time has not passed for the oxytocin to take effect; 2) improper or inexperienced milking technique; 3) physical differences in the nipple; 4) incorrect intraperitoneal injection of the oxytocin. The researcher may therefore wait a few more minutes prior to attempting to milk again; if this does not result in increased milk yield, the researcher may choose to move to a different milking site. Using a vacuum, each set of teats (upper thoracic, lower thoracic, upper abdominal, and lower abdominal) was equally successful in expelling milk when the milker is proficient, and approximately 0.5 ml can be obtained from each teat<sup>12</sup>. Therefore, if one milking site is proving difficult, moving to another site may help. The authors are not aware of differences in milk composition between milking sites. Finally, a second dose of oxytocin may be administered to further stimulate milk letdown. Diluting the oxytocin to a standard dose volume/kg body weight can also be attempted, for example 1 ml/kg body weight<sup>12</sup>, however be aware of maximum intraperitoneal injection volumes.

It is also possible that other factors, including but not limited to, rodent strain, health status, parity, litter number, level of stress, diet, time of day, and food intake may affect the outcome of the milk collection. While the discussion of all these variables is beyond the scope of this protocol, it is encouraged that the investigators consider the multitude of factors that may influence the efficiency of milk collection in their experiments.

Finally, a simple method of measuring the proportion of cream in the milk, the creatocrit, which is an accurate estimation of the amount of lipid in the samples<sup>11</sup>, is described herein. The creatocrit can then be used to estimate the fat concentration and energy value of the milk. Though there were no differences in creatocrit, fat concentration, energy value, or protein concentrations between the maternal dietary groups in the results presented above, others have found differences in creatocrit values based on maternal characteristics such as gestational age<sup>21</sup> and duration of lactation<sup>22</sup>. Differences in oligosaccharide content in maternal milk has also been demonstrated with maternal dietary manipulation<sup>6</sup>.

In summary, milk contains a plethora of bioactive compounds whose specific roles in early life development of offspring, and their subsequent effect on disease susceptibility throughout life, remain to be clarified. The protocol reported here allows milk to be collected from lactating rats by a single individual, requires no specialized vacuum apparatus, and provides sufficient milk for subsequent analysis. Mastering this technique will provide the researcher with the opportunity to contribute significant insight into the processes involved in early-life development and establishment of health.

## Disclosures

The authors have no conflicts of interest to disclose. All animal experiments were conducted in accordance with CCAC approved protocols.

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